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Lymphocytopathogenic activity in vitro correlates with high virulence in vivo for BVDV type 2 strains: Criteria for a third biotype of BVDV

Julia F. Ridpath [a,*], Stefanie Bendfeldt [b], John D. Neill [a], Elisabeth Liebler-Tenorio [c]

[a] Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, IA 50010, USA

[b] Institute of Virology, School for Veterinary Medicine Hannover, Hannover, Germany

[c] Federal Research Institute for Animal Health, Jena, Germany

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Abstract

Two biotypes of bovine viral diarrhea viruses (BVDV), cytopathic (cp) and noncytopathic (ncp), are recognized based on their activity in cultured epithelial cells. Biotype does not correlate to virulence in acute infections as BVDV strains associated with severe acute BVD outbreaks are all noncytopathic based on their growth characteristics in cultured epithelial cells. Previous studies have shown that acute infections with highly virulent BVDV result in depletion of cells in lymphoid tissues. In this study, flow cytometry demonstrated that infection with a highly virulent BVDV strain was associated with a pronounced reduction in circulating white blood cells (WBC) and increased numbers of apoptotic and necrotic circulating WBC in vivo. Infection with low virulence BVDV did not result in a significant increase in death of circulating WBC. Thus, there appeared to be a correlation between depletion of circulating WBC and virulence. To study the interaction of BVDV strains with lymphoid cells in the laboratory, we developed an in vitro model that used a bovine lymphoid cell line (BL-3 cells). Using this model, it was found that while BVDV strains are segregated into two biotypes based on their activity in cultured epithelial cells, they may be segregated into three biotypes based on their activity in cultured lymphoid cells. These three biotypes are noncytopathogenic (no obvious effects on the viability of either cultured epithelial or lymphoid cells), cytopathogenic (cytopathic effect and cell death in both cultured epithelial and lymphoid cells within 48 h of infection) and lymphocytopathogenic (no effect on cultured epithelial cells, however, cell death in cultured lymphoid cells is observed within 5 days of infection). The proposed lymphocytopathic biotype correlates with high virulence in acute infections in vivo. Cell death caused by the lymphocytopathogenic biotype was not associated with changes typically seen with cytopathic viruses grown in cultured epithelial cells (e.g. changes in processing of the NS2/3 protein observed within 24 h post infection, crenation and breakdown of cell integrity within the first 48 h post infection). These data suggest that the cytopathic effect induced in cultured lymphoid cells by a ncp highly virulent BVDV strain may occur by a different mechanism than the cytopathic effect induced by cp BVDV strains.
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Keywords: Bovine viral diarrhea virus; Biotype; Virulence in vivo; Cytopathogenic; Noncytopathogenic; Lymphocytopathogenic

1. Introduction

Bovine viral diarrhea viruses (BVDV) are endemic in ruminant populations worldwide. Like other members of the pestivirus genus, within the Flavivirus family, they are small enveloped viruses with a single stranded RNA genome (Gillespie et al., 1960; Heinz et al., 2000; Lee and Gillespie, 1957; Lindenbach and Rice, 2001). Two genotypes and two biotypes of BVDV have been recognized (Gillespie et al., 1960; Lee and Gillespie, 1957; Pellerin et al., 1994; Ridpath et al., 1994). The two genotypes are called BVDV1 and BVDV2 and are now recognized as distinct species within the pestivirus genus (Heinz et al., 2000). The two biotypes, cytopathogenic and noncytopathogenic, are based on the activity of the BVDV strain in cultured epithelial cells (Gillespie et al., 1960; Lee and Gillespie, 1957). The practical significance of biotype is that, in vivo, noncytopathogenic viruses may establish persistent infections following in utero infection but cytopathogenic viruses do not. Noncytopathogenic viruses predominate in nature. Cytopathogenic viruses are relatively rare and usually found in association with outbreaks of mucosal disease, a relatively infrequent but highly fatal form of BVDV infection (Houe, 1995, 1999, 2003;

* Corresponding author. Tel.: +1 515 663 7586; fax: +1 515 663 7458.
E-mail address: jridpath@nadc.ars.usda.gov (J.F. Ridpath).

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Lindberg, 2003). Cytopathology in vitro does not correlate with virulence in vivo (Bezek et al., 1994) and the most clinically severe form of acute BVDV infection is associated with non-cytopathogenic virus (Bolin and Ridpath, 1992; Carman et al., 1998; Corapi et al., 1990, 1989; Pellerin et al., 1994; Ridpath et al., 1994).

The effects of acute infection with BVDV may range from clinically unapparent to clinically severe. Clinical presentation following acute infection is dependent on the viral strain and immune status of the animal. Clinically severe BVDV, also known as severe acute BVDV (SA BVDV), occurs in animals infected with a type 2 BVDV that have no or low titers against BVDV2 strains. It is associated with a greater than 50% reduction in circulating lymphocytes and platelets and body temperatures exceeding 40.6 °C (Carman et al., 1998; Liebler-Tenorio et al., 2002, 2003b). Severe acute BVD may progress to hemorrhagic syndrome in some cases (Corapi et al., 1990, 1989; Stoffregen et al., 2000). Viral antigen may be detected in epithelial cells following infection in vivo, however, the principle site of replication and spread of the virus is in lymphoid tissue (Liebler-Tenorio et al., 2002, 2003a,b). While both clinically severe and subclinical BVDV infections are associated with lymphoid depletion, the depletion seen with highly virulent strains is more extensive and longer in duration (Liebler-Tenorio et al., 2002, 2003a,b). Although lymphoid tissue is a major replication site of the virus in vivo and pathogenesis is associated with depletion of lymphoid tissues, most in vitro studies have been done using cultured epithelial cells because of the greater availability of epithelial cell lines.

Initial studies detailed here compared the in vivo effects of infection following infection with two type 2 noncytopathogenic BVDV with different levels of expressed virulence. While infection with one virus, BVDV2-RS866, resulted in subclinical or mild clinical signs, infection with the other virus, BVDV2-1373, resulted in severe clinical disease. We attempted to develop an in vitro model to study the differences in virulence displayed by these two viruses. To this end, we compared the results of infection on cultured epithelial and lymphoid cells lines in vitro. While we saw no difference between the two viruses in cultured epithelial cells, we observed a decrease in replicating cells in cultured lymphoid cell lines. To determine if this cell death was similar to the cytopathology observed with cytopathic BVDV strains, we included a cytopathic strain from the same genotype, BVDV2-296c, in the cultured lymphocyte studies. While infection with both BVDV2-1373 and BVDV2-296c resulted in cell death in BL-3 cultures, the time lapse between infection and cell death and the pathway involved in cell death were different.

2. Materials and methods

2.1. Isolation, characterization and propagation of viruses

The BVDV2 strains used in this study originated in the United States or Canada and were isolated between 1993 and 1998. Strain BVDV2-1373 was isolated from a severe acute BVDV outbreak in Ont., Canada (Carman et al., 1998). This strain reproducibly causes severe acute disease in calves seronegative to BVDV (Liebler-Tenorio et al., 2002; Stoffregen et al., 2000). The noncytopathic strains BVDV2-28508-5 and BVDV2-RS886 were isolated from persistently infected asymptomatic calves. Infection with either of these strains results in a subclinical infection (Liebler-Tenorio et al., 2003a, 2004; Ridpath et al., 2000). The cytopathic/noncytopathic pair BVDV2-296c and BVDV2-296nc was isolated from a mucosal disease case (Ridpath and Neill, 2000). Strains were assigned to BVDV genotype 2 based on phylogenetic analysis of the 5′ UTR region (Ridpath et al., 1994). Strains were assigned to the cytopathogenic or non-cytopathogenic biotype based on activity in cultured bovine epithelial cells (Gillespie et al., 1960) and production of NS3 (Donis and Dubovi, 1987; Pocock et al., 1987) as determined by radioimmunoprecipitation using bovine polyclonal antisera (Ridpath and Bolin, 1990) as described in previous publications (Liebler-Tenorio et al., 2003a, 2004; Ridpath et al., 1994, 2000). Viruses were propagated as described earlier (Ridpath et al., 2002) with the exception that the Madin Darby bovine kidney (MDBK) cell line was used rather than bovine turbinate cells. Fetal bovine serum used to supplement cell culture medium was tested free of BVDV and antibodies against BVDV (Bolin et al., 1991b).

2.2. Animal model

Mixed breed calves were caught at birth and fed milk replacer that was tested free of BVDV and antibodies to BVDV. All calves tested negative for BVDV at birth, as determined by virus isolation from buffy coat samples followed by detection based on polymerase chain reaction (PCR) assay and immunohistochemistry staining for the presence of BVDV antigens in skin (Ridpath et al., 2002). Virus isolation from buffy coat samples was also performed on samples collected immediately preceding virus inoculation to assure that animals were free of circulating BVDV at the time of inoculation. In addition, calves were tested free of antibodies against BVDV at birth and immediately before inoculation with virus as determined by serum neutralization using BVDV type-1 strain BVDV1-NY-1 and BVDV type-2 strain BVDV2-1373 (Ridpath et al., 2002).

Age at inoculation ranged from 2 to 9 months. Nine calves were infected with BVDV2-1373 and five were infected with BVDV2-RS886. Four calves served as noninfected controls. Animals were infected with 5 ml of inoculum [titer of $1 \times 10^6\,\mathrm{ml}^{-1}$ tissue culture infectious dose (TCID)] by the oral/nasal route. Temperatures were taken daily. Blood samples for determination of white blood cell (WBC) counts were collected on days 0, 2, 4, 6, 9, 11 and 13 post inoculation. The WBC counts were determined using an HV 1500 cytometer (CDC Technologies, Inc., Oxford, CT) per manufacturer's directions. Buffy coat samples were collected on days 0, 3 or 4, 9 and 13 days post infection.

Blood was collected in buffered sodium citrate on days 0, 2, 4, 6, 9, 11 and 13 days post infection for flow cytometric analysis of dead (propidium iodide uptake) and apoptotic (annexin binding) circulating WBC. Propidium iodide uptake and annexin binding were done using a TACS Annexin V kit (Trevigen, Inc., Gaithersburg, MD) and processed for flow cytometry per manufacturer's directions. Unstained cells and cells from noninfected calves were run as controls. Flow cytometry was performed using a BD FacScan (Becton Dickinson, San Jose, CA). Ten thousand events were analyzed for each sample. Flow cytometry data was collected and analyzed using the Cell Quest software package (Becton Dickinson).

2.3. Cell culture model

The in vitro model was based on the BL-3.1 cell line (ATCC #CRL-2306) which is a nonadherent cell line available from the American Type Culture Collection (ATCC, Manassa, VA). The original cell line was derived from a Hereford calf with a B cell lymphosarcoma (Theilen et al., 1968). The variant of the original cell line used in this study does not produce the bovine leukemia virus (Harms and Splitter, 1992) but as provided by the ATCC is contaminated with a BVD virus. For the purposes of this study, the BL-3 cell line was cleared of BVDV by cloning via limiting dilution. Clones were tested free of BVDV by direct immunohistochemistry, direct RT-PCR and by immunohistochemistry and RT-PCR following two blind passages of cell lysates on MDBK cells. This cell line will be referred to hereafter as BL-3 cells. BL-3 cells were retested for BVDV prior to and during each experiment. BL-3 cells were propagated as suspension cultures in Leibovitz's L-15 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 100 μg/ml gentamicin (GentaMax 100, AmTech Group Inc., St. Joseph, MO). The fetal bovine serum used was tested free of BVDV and antibodies against BVDV (Bolin et al., 1991a). Cells were maintained at a density between 1×10^5 and 1×10^6 cells/ml as recommended by the ATCC. BL-3 cells were maintained in a humidified incubator at 37 °C and 5.5% CO_2. The protocol used for virus inoculation was as follows. Approximately, 1×10^6 cells were removed from a suspension culture and spun down ($910 \times g$, for 20 min) and resuspended in 1 ml of viral inoculum (1×10^6 TCID/ml in complete cell medium). The resulting cell suspension was rocked at 37 °C for 1 h. At the end of this time period, the cells were spun down (as described above), the supernatant discarded and 5 ml of complete cell medium added.

Cell counts were determined using a Coulter T-890 counter per manufacturer's directions (Coulter, Hialeah, FL). For staining protocols, suspension cells were spun down onto ProbeOn Plus microscope slides (Fisher Biotech, Pittsburgh, PA) using a Cytospin 3 Cell Preparation System (Shandon Scientific Limited, Cheshire, UK). Slides were immediately fixed for 5 min in −80 °C 100% methanol and air dried. Fixed slides were stored at −80 °C until use. Cell proliferative state was assessed by detection of Ki67 antigen using the monoclonal antibody MTB1 as described by Liebler-Tenorio and Pohlenz (1997). Viral antigen was detected via immunohistochemistry using a monoclonal antibody that recognized the E2 viral protein as described previously (Liebler-Tenorio et al., 2003b). Radioimmunoprecipitation as described in earlier studies (Bolin and Ridpath, 1989; Ridpath and Bolin, 1990) was performed using polyclonal sera collected from convalescent cattle. The ratio of live to dead cells was based on flourescein diacetate (FDA) uptake (live cells) versus propidium iodide uptake (dead cells). The ratio was determined as follows. Stock solutions of FDA (12 mM FDA in acetone) and propidium iodide [1 mg/ml in PBS (14.5 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 145.4 mM NaCl)] were stored in the dark. Immediately before use propidium iodide solution was diluted 1:14.3 with PBS and the FDA stock was diluted by adding 2 μl of FDA stock to 5 ml of PBS. Cell cultures were inverted and swirled to insure an even distribution of cells, a 1 ml sample removed and 100 μl each of FDA and propidium iodide solution were added. Samples were analyzed by flow cytometry within 5 min of addition of FDA and propidium iodide solutions. Flow cytometry was performed using a BD FacScan (Becton Dickinson). Ten thousand events were analyzed for each sample.

2.4. Preparation of BL-3 cell extracts for Western Blot analysis

Aliquots of 10^7 cells were seeded in 25 cm^2 flask at a total volume of 5 ml. Flasks were positioned upright in the incubator for the duration of all experiments. The cells were infected at a multiplicity of infection (MOI) of 1 and fed daily by replacement of at least 1 ml of media.

Samples of BVDV2-1373 and BVDV2-28508 infected cells samples were collected daily, samples of BVDV2-296c infected cells were collected at 3, 6, 12, 18 and 24 h post inoculation. Aliquots of BL-3 cell cultures containing 4×10^7 cells were removed and cells pelleted by centrifugation ($910 \times g$, for 20 min). Cells were resuspended in ice-cold PBS and pelleted by centrifugation ($910 \times g$, for 20 min). The cell pellet was resuspended in 300 μl ice-cold lysis buffer containing protease inhibitors and phosphatase inhibitors per manufacturer's directions. Lysis buffer was purchased from (Cell Signaling Technology, Beverly, MA) and the phosphatase inhibitors were purchased from Sigma–Aldrich (St. Louis, MO). The protease inhibitor cocktail Complete was purchased from Roche (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Following the addition of lysis buffer, samples were sonicated for 10 s. After centrifugation at 4 °C for 15 min at $14{,}000 \times g$, the supernatant containing the protein fraction was stored at −80 °C until use. Protein concentration was determined using the BCA Protein Reagent Assay (Pierce Biotechnology, Inc., Rockford, IL).

2.5. SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE using 7.5% acrylamide gels. Prestained molecular weight markers (SeeBlue Plus 2, Invitrogen, Carlsbad, CA) were included on each gel. After electrophoresis, proteins were transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). After blocking with blocking reagent (Amersham Biosciences), the membranes were incubated with the appropriate antibodies. Incubations with primary antibodies were overnight at 4 °C. Incubations with secondary antibodies were for 1 h at room temperature. As a control for total protein concentration, the lower part of the membrane was cut and stained for actin expression. Detection of proteins was performed using BM chemiluminescence blotting substrate. The following antibodies were used: anti-

PARP (BD Biosciences Pharmingen, San Diego, CA), anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), horse anti-goat HRP (Vector Laboratories, Burlingame, CA) and horse anti-mouse HRP (Vector Laboratories). The BM chemiluminescence blotting substrate was purchased from Roche (F. Hoffmann-La Roche Ltd.).

3. Results

3.1. Infection in vivo

The viability and number of circulating WBC were examined following infection of calves with a high virulence BVDV2 strain (BVDV2-1373) or lower virulence strain (BVDV2-RS886). Criteria observed were the number of circulating WBC per milliliter and the percentage of circulating WBC that bound annexin and/or took up propidium iodide (necrotic plus apoptotic cells). All animals replicated virus as evidenced by the isolation of virus from buffy coat samples (data not shown). Decreases from baseline level of WBC was observed in calves infected with both viruses (Fig. 1a). There were increases in the percentage of apoptotic and necrotic circulating WBC as compared to baseline levels in all animals (Fig. 1b). However, these increases are significantly higher in animals infected with BVDV2-1373 on day 4, 6 and 9 post inoculation. Animals infected with the high virulence virus were not just loosing circulating WBC, many of the remaining cells were dead or in the process of dying. Thus, infection with the high virulence virus correlated with death of circulating WBC.

3.2. Infection in vitro

No significant differences in growth rate were observed between noninfected BL-3 cells and BL-3 cells infected with either the high virulence virus BVDV2-1373 or the low virulence virus BVDV2-28508-5 at 2 days post infection (Fig. 2). However, 5 days after infection the growth rate seen in BVDV2-1373 infected cells was significantly lower. This difference in growth rate did not result in a difference in the amount of virus present, as there was no significant difference in viral titers found in freeze/thaw lysates harvested after 5, 6 and 7 days post infection (Table 1). To determine if the reduced growth rate was due to a slowdown in cell replication or a reduction in number of replicating cells, samples of cells were removed 1, 2, 3, 4, 5, 6 and 7 days post infection and assayed for expression of Ki67 (Fig. 3). Proliferating cells produce Ki67, a protein associated with proliferating or dividing cells. The BVDV type 2 cytopathic strain BVDV2-296c was included for comparison. Two days post infection the majority of cells in control cultures and cultures infected with BVDV2-28508-5 and BVDV2-1373 are in a proliferative state. In contrast most of the cells in cultures infected with BVDV2-296c are in a nonproliferative state. By day 5, there were fewer proliferating cells in BL-3 cultures infected with BVDV2-1373 than in either control cells or cells infected with BVDV2-28508-5. The number of proliferating cells continued to decline though the last time point tested, day 7. At this time point very few proliferating cells were found. In contrast the number of proliferating cells seen in BVDV2-28508-5 infected cells appeared similar to that of noninfected control cells.

Table 1
Titer of virus (log 10 ml^{-1}) isolated from freeze/thaw lysates of BL-3 cells infected with the high virulence virus BVDV2-1373 or the low virulence virus BVDV2-28508-5

	Day 5	Day 6	Day 7
BVDV2-1373 infected	6.6	6.4	7.2
BVDV2-28508-5 infected	6.8	6.4	6.8

Values are averages of three replicates.

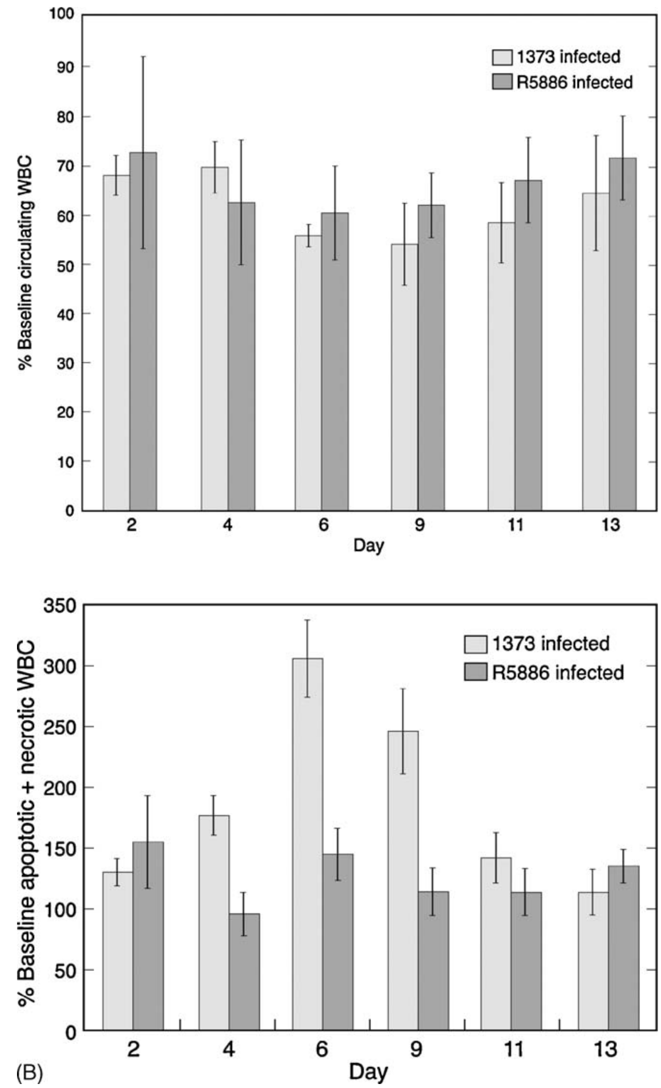


Fig. 1. In vivo infections of high virulence or low virulence BVDV. The percent of WBC per milliliter blood compared to preinoculation levels (baseline values) for animals infected with a high virulence BVDV (BVDV2-1373) and a lower virulence BVDV (BVDV2-RS886), from 2 to 13 days post infection is shown in panel a. Panel b shows the percent of circulating WBC that bind annexin (apoptotic state) and/or take up propidium iodide (necrotic state) compared to baseline values. The results shown represent nine calves infected with BVDV2-1373 and five calves infected with BVDV2-RS886. Error bars represent standard error of the mean.

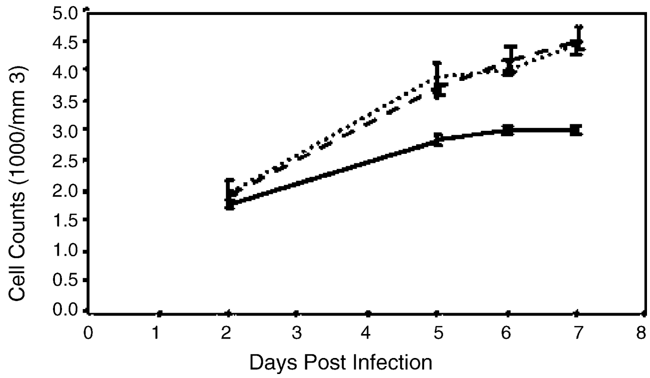


Fig. 2. Growth rates of BL-3 cultures following infection with BVDV strains. The concentration of cells over time in BL-3 suspension cultures following infection with the highly virulent virus BVDV2-1373 (—) and the low virulence virus BVDV2-28508-5 (- - -). Values for noninfected cultures are also shown (· · ·). These graphed values represent the average of three replicates. Error bars represent standard error of the mean.

Table 2
Ratio of live to dead cells at 2 and 7 days post infection as determined by FDA uptake versus propidium iodide uptake

	Day 2	Day 7
Control	2.8 ± 0.02	5.75 ± 3.17
BVDV2-296c	0.54 ± 0.11	0.31 ± 0.02
BVDV2-1373	2.67 ± 0.33	0.43 ± 0.02
BVDV2-28508-5	2.61 ± 0.41	7.28 ± 3.07

Values are averages of three replicates.

BL-3 cells infected with BVDV2-296c evidenced cytopathic effect characterized by crenation and breakdown of cell integrity (Fig. 4a). BL-3 cells infected with BVDV2-1373 while slowing in growth and entering a nonproliferative state did not show evidence of a similar cytopathic effect (Fig. 4b). To determine whether BL-3 cells infected with BVDV2-1373 were dead or in a quiescent state the ratio of live to dead cells over time was determined. The results of these studies are summarized in Table 2. Two days after inoculation the majority of BL-3 cells infected with BVDV2-296c exhibited cytopathology, were in a nonproliferative state and stained with propidium iodide. In contrast, at day 2, there was no statistical difference between control cells and cells infected with BVDV2-1373 or BVDV2-28508-5. However, by day 7, most of the cells infected with BVDV2-1373 were in a nonproliferative state and stained with propidium iodide. These cells did not re-enter a proliferative state following refeeding or passage (data not shown).

To examine the mechanism of cell death PARP cleavage was examined in noninfected BL-3 cultures and cultures infected with BVDV2-296c, BVDV2-1373 and BVDV2-28508-

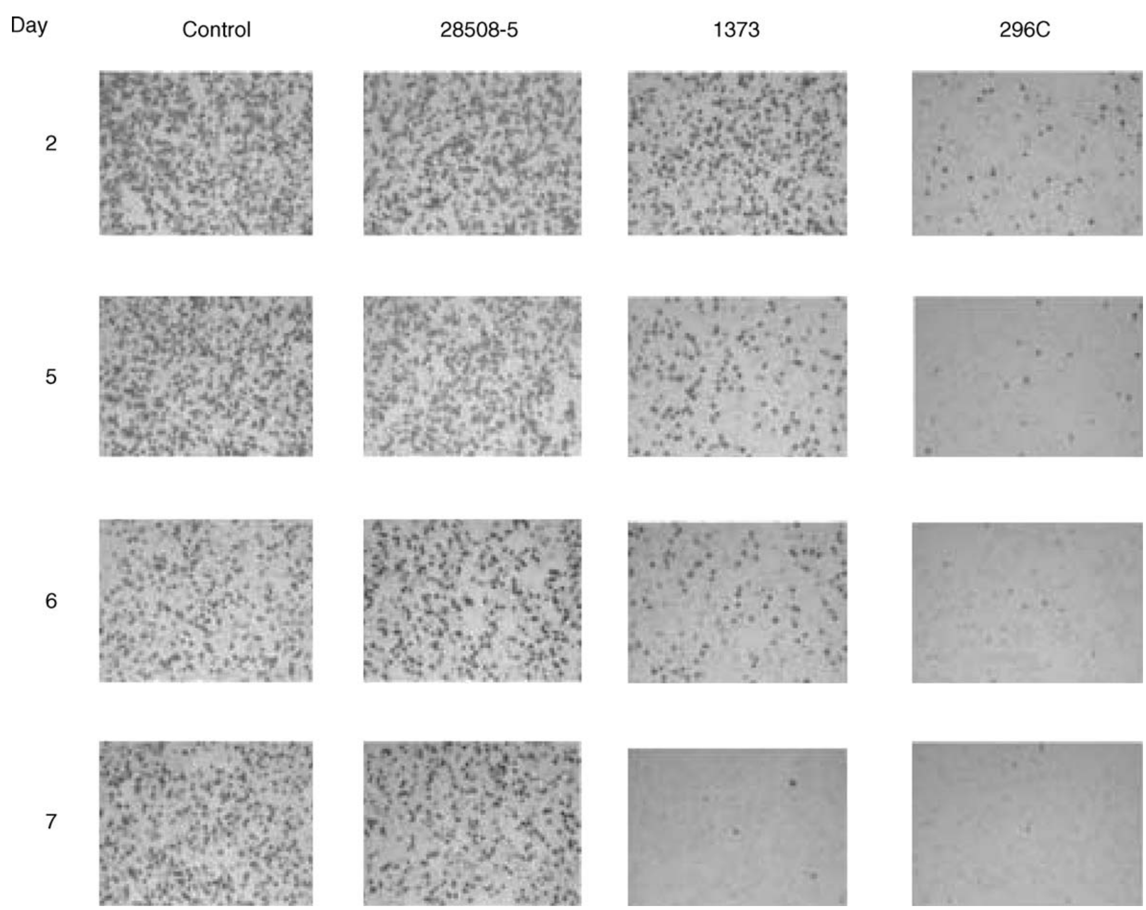
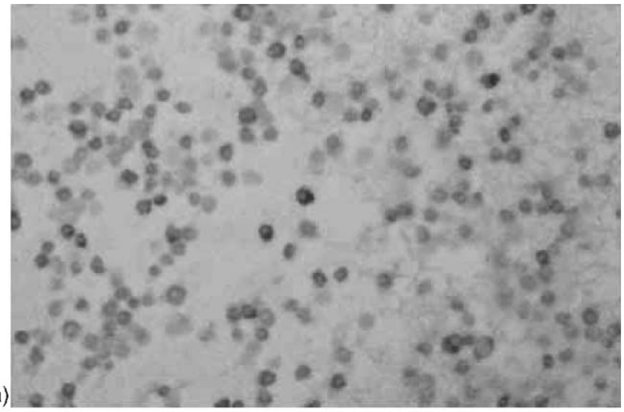
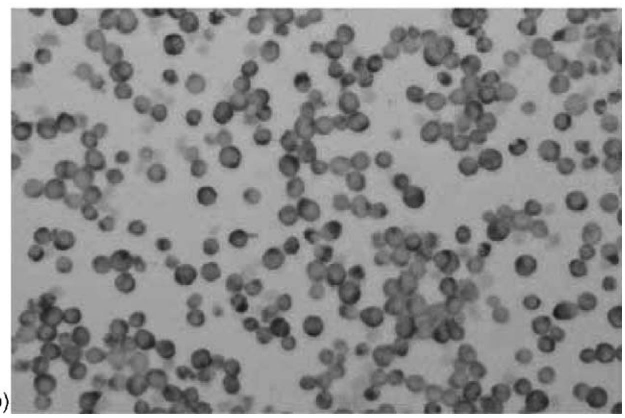


Fig. 3. Proliferative state, as determined by Ki67 expression, in BL-3 cultures infected with BVDV strains. Aliquots of cells from noninfected BL-3 cultures and cultures infected with the noncytopathic low virulence virus BVDV2-28508-5, the noncytopathic highly virulent virus BVDV2-1373 and the cytopathic low virulence virus BVDV2-296c were assayed for Ki67 expression. Proliferating cells will express Ki67 in the nucleus resulting in staining of nuclei in this assay.



(a)



(b)

Fig. 4. Morphology of BL-3 cells infected with different BVDV strains. Binding of the a Mab produced against the BVDV protein, E2 in shown in cells from BL-3 suspension cultures harvested 24 h after infection with the low virulence cytopathic BVDV BVDV2-296c (panel a) or 5 days after infection with the high virulence noncytopathic BVDV BVDV2-1373 (panel b). BL-3 cells infected with BVDV2-296c evidenced cytopathic effect characterized by crenation and breakdown of cell integrity. BL-3 cells infected with BVDV2-1373 while slowing in growth and entering a nonproliferative state did not show evidence of a similar cytopathic effect.

5 (Fig. 5). It is well established that cleavage of PARP is a hallmark of apoptotic cells where the PARP molecule is cleaved by activated caspase 3. No cleavage was observed in control cells or BVDV2-28508-5 cells. There was extensive PARP cleavage in cells infected with BVDV2-296c, in agreement with a previous report for cell death in bovine cells infected with cytopathic strains of BVDV (Hoff and Donis, 1997). In contrast there was no PARP cleavage at early time points and very limited PARP cleavage at later time points in cells infected with BVDV2-1373. Time points past 7 days were not examined as it appeared most cells were dead as determined by propidium iodide uptake.

4. Discussion

All highly virulent type 2 strains reported to date have been characterized as noncytopathic based on their activity in cultured epithelial cells (Bolin and Ridpath, 1992; Carman et al., 1998; Corapi et al., 1990, 1989). Cattle infected with noncytopathic BVDV exhibited reduced numbers of circulating WBC. Reduction of circulating WBC is characteristic of acute infections with most pestiviruses (Heinz et al., 2000). The level of reduction observed with noncytopathic BVDV is dependent on the viral strain, with the most virulent virus causing the greatest decrease in numbers. Decreased numbers of WBC may be the result of trafficking from blood into tissue, a reduction in leukogenesis or outright death of WBC. The high percentage of apoptotic and/or necrotic WBC observed in animals infected with a high virulence BVDV strain suggest that the reduction may be due to cell death. This in vivo observation is supported by the in vitro observation that infection, of a cultured lymphoid cell line, with a high virulence virus leads to cell death. The mechanism of cell death was not defined by these studies. However, it appears to be different than the mechanism that induces apoptosis in cells infected with cytopathic BVDV. BL-3 cells infected with a cytopathic virus exhibit cytopathic effects within 24 h and most cells are dead within 48 h. In contrast, BL-3 cells infected with a noncytopathic high virulence virus do not exhibit the cytopathic effect observed with a cytopathic

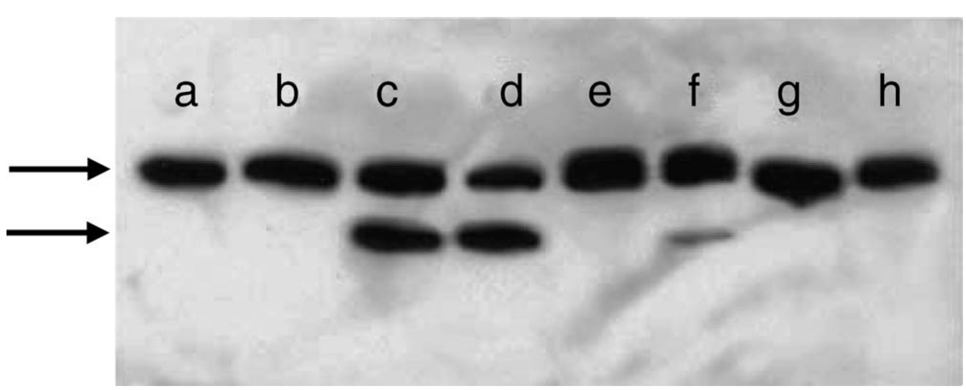


Fig. 5. PARP cleavage following infection with different BVDV strains. PARP cleavage was examined in western blots of lysates of noninfected BL-3 culture, and cultures infected with BVDV2-296c, BVDV2-1373 and BVDV2-28508-5. Lanes a and b represent lysates made from a 2- and 5-day-old noninfected BL-3 culture, respectively, lanes c and d represent lysates made from BL-3 cultures 18 and 24 h after infection with the low virulence cytopathic virus BVDV2-296c, lanes e and f represent lysates made from cultures 2 and 5 days post infection with the highly virulent, noncytopathic virus BVDV2-1373 and lanes g and h represent lysates made from BL-3 cultures 2 and 5 days post infection with the low virulence, noncytopathic virus BVDV2-28508-5. The upper arrow points to uncleaved PARP while the lower arrow points to the cleaved form.

virus and do not have significantly higher numbers of dead cells, compared to noninfected controls, at 48 h. The number of dead cells observed in BL-3 cultures infected with a high virulence virus does not become significant until 4–5 days post infection. It is not just that cell death is delayed compared to infection with cytopathic virus. It appears that there is a different mechanism in effect as evidenced by the difference in cleavage of PARP. While the ratio of live to dead cells, observed 5 days after infection with the noncytopathic high virulence virus, was similar to that seen in BL-3 cells infected with a cytopathic BVDV, the level of PARP cleavage differed. PARP cleavage in the BL-3 cells infected with the noncytopathic high virulence BVDV was minor compared to that of BL-3 cells infected with a cytopathic virus. The cell death observed in BL-3 cells infected with the high virulence virus was not seen in cells infected with the low virulence virus, thus correlating activity in vitro with virulence in vivo. From these results, it appears that infection of BL-3 cells may be used as a model to study virulence in vitro. Lymphoid cell death was not associated with increased levels of virus replication as equivalent titers of virus were recovered after infection with high virulence or low virulence strains. The exact mechanism at work in causing death in lymphoid cells will be a subject of future research in our laboratory.

These observations lead us to the conclusion that more than two biotypes can be identified among BVDV strains. Currently, two biotypes of BVDV, cytopathic and noncytopathic, are recognized based upon the activity of a virus in cultured epithelial cells. Previous research, in numerous laboratories, have examined differences between cytopathic and noncytopathic BVDV strains. However, the differences observed between strains from different biotypes did not correlate with clinical signs following infection in vivo. Strains that caused severe acute BVDV, which is characterized by wide spread destruction of lymphoid tissue in vivo, did not cause cytopathology in cultured epithelial cells. Strains that caused severe acute disease, similar to low virulence noncytopathic strains, did not cleave the viral NS2/3 into NS2 and NS3. In fact, in cultured epithelial cells, there were no distinguishable differences between high virulence and low virulence viruses. In contrast, the difference between low virulence and high virulence BVDV is easily discernable in cultured lymphoid cells. Within 5 days, at a M.O.I. of one, infection with a high virulence virus will result in nearly 100% die off of cultured lymphoid cells. Infection with a low virulence virus will have no apparent affect on cell health or replication. The cell death observed with high virulence BVDV is different in timing and mechanism from that observed with cytopathic BVDV strains. These results suggest that there are three biotypes of BVDV. Noncytopathic BVDV strains (noncytopathic biotype) do not cause cell death in either cultured epithelial or lymphoid cells, cytopathic BVDV strains (cytopathic biotype) cause cell death within 48 in either cultured epithelial or lymphoid cells and lymphocytopathic BVDV strains (lymphocytopathic biotype) that while not causing cell death in cultured epithelial cells will cause cell death in cultured lymphoid cells. The practical significance of the lymphocytopathic biotype is that it correlates with virulence in vivo. Further the cultured lymphoid cell line model has the potential to be a valuable tool in understanding the effects of BVDV infection on the immune system. However, further research needs to be done with other highly virulent pestiviruses and other types of lymphoid cells to determine if these observations are universal to all highly virulent pestiviruses.

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